Docket No.: 19036/37157
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Nobutaka Wakamiya

Application No.: 09/763,712

Filed: May 4, 2001 (§371)
Aug. 24, 1999 (PCT)

For: Polynucleotides Encoding Human Collectin and Polypeptides Encoded Thereby

Group Art Unit: 1636

Confirmation No. 9190

Examiner: Daniel M. Sullivan

CERTIFICATE OF MAILING

I hereby certify that this paper is being deposited with the U.S. Postal Service as First Class Mail in an envelope, postage prepaid, addressed to Mail Stop Amendment Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on August 6, 2004.

Kim A. Brace

37 C.F.R. § 1.132 DECLARATION OF NOBUTAKA WAKAMIYA

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Nobutaka Wakamiya, do hereby declare and state as follows:

INTRODUCTION

1. I am the inventor of the subject matter claimed in U.S. Patent Application No. 09/763,712. My current CV is attached.

2. Experiments were performed to confirm the properties of the human collectin described in the specification. Collectins play a role in innate immunity by binding the carbohydrates (saccharides) on the surface of various pathogenic microbes. As innate immune system molecules, the collectins do not necessarily possess the level of specificity of the immunoglobulins of the adaptive immune system. However, the collectins do have specificity as they are able to distinguish microbial saccharides from host glycoproteins.

3. The collectin described in the present application is a C-type lectin. C-type lectins bind saccharides in a Ca^{2+}-dependent manner. Accordingly, in order to demonstrate that the claimed collectin not only binds saccharides, but binds them in a Ca^{2+}-dependent manner, saccharide binding assays were performed in both the presence and the absence of a Ca^{2+} chelator (EDTA). Before performing the assays, the collectin polypeptide was first prepared.

PREPARATION OF HUMAN COLLECTIN POLYPEPTIDE OF 389TH-547TH AMINO ACIDS IN SEQ ID NO:2

4. The polypeptide tested was a recombinant collectin including a carbohydrate recognition domain (CRD), a neck region and six continuous amino acids (two units of a Glycine-X-Y sequence (wherein X and Y are optional amino acids)) at the C-terminus in a collagen-like region. The polypeptide was expressed in *Escherichia coli.* Genes to be introduced into an expression vector were amplified from a human placenta cDNA library using PCR.

5. Expression of the polypeptide in *Escherichia coli* was performed as follows. N-terminal primer (5'-CGCGGATCCGGCCCATCAGGAGCGGTGGTG-3') was prepared by combining eighteen bases encoding six amino acids at the C-terminus in a collagen-like region with those encoding the precedent *BamH* I restriction site. C-terminal primer (5'-CCGGAATTCTTATAATGCAGATGACAGTA-3') was prepared by combining the bases encoding the termination codon at the C-terminus of the subject polypeptide with those encoding the successive *EcoR* I restriction site. Fragments were amplified with a PCR program of 35 cycles wherein a cycle includes denaturation at 98°C for 10 seconds, annealing at 68°C for 5 minutes and extension at 68°C for 5 minutes. Amplified fragments and the expression vector pRSET-C (InvitroGene) were digested respectively with the restriction enzymes *BamH* I and *EcoR* I. Upon purifying the digested fragments and the vector, they were ligated to construct a vector for expression of the polypeptide to be tested. The vector incorporates a six histidine tag at the C-terminus of the sequence to be expressed. The expression vector was transformed into competent cells of *Escherichia coli* BL21(DE3)pLysS (InvitroGene). Bacteria were collected after confirming expression. The collected bacteria were then solubilized with TBS/C (10mM Tris-HCl, 150mM NaCl, 5mM $CaCl_2$) containing 6M urea and the polypeptide was purified through an affinity column of

nickel-nitrilotriacetic acid agarose (QIAGEN). Fractions of purified polypeptide were dialyzed using a TBS/C solution.

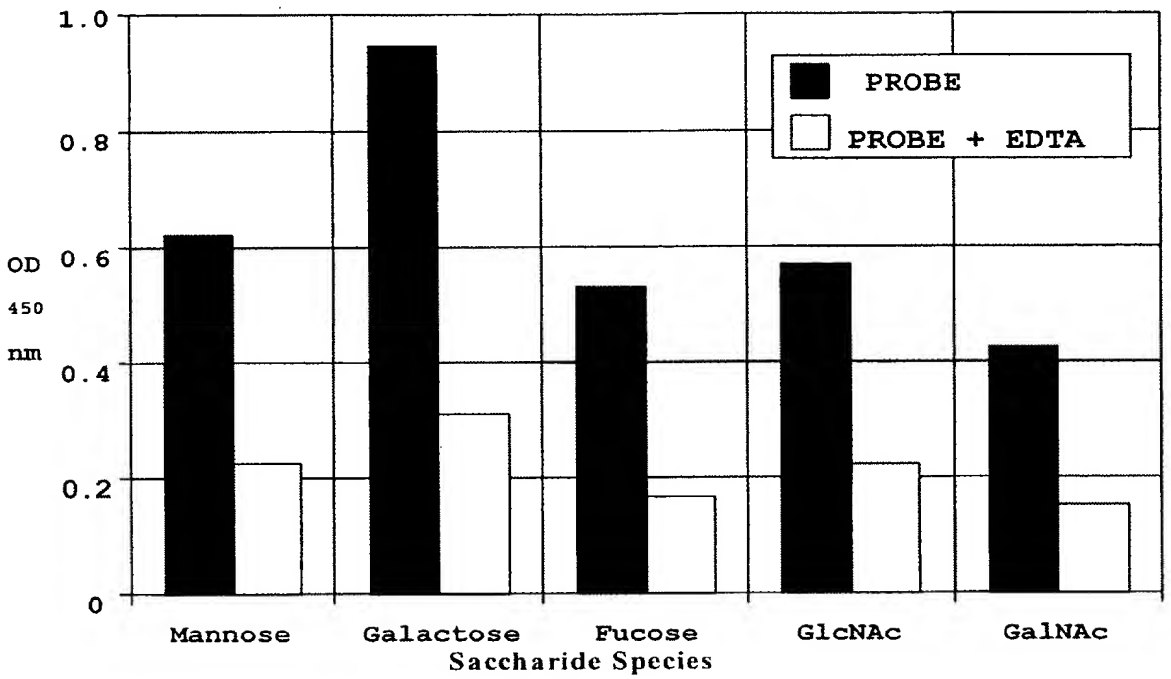
EVALUATION OF BINDING ACTIVITIES ON POLYSACCHARIDES BY HUMAN COLLECTIN POLYPEPTIDE OF 389TH-547TH AMINO ACIDS IN SEQ ID NO:2

6. Microtiter-plates were coated overnight at 4°C by emersion in 100μl coating buffer (15mM sodium carbonate, 35mM sodium hydrogencarbonate, 0.05% sodium azide, pH9.6) containing the purified polypeptide (*i.e.*, the recombinant collectin; 10μg/ml). Coated plates were washed three times with TBS/TC (10mM Tris-HCl, 150mM NaCl, 5mM CaCl2, 0.05% Tween20®) and were blocked by incubating them with BlockAce (Dainippon Pharmaceutical CO., LTD.) at room temperature for one hour.

7. Binding between the polypeptides on the plates and biotinylated saccharide probes (Seikagaku Corporation) was determined. As a first step, 100μl of the reagent solution prepared by combining any of 10μg/ml Mannose-BP/TBSC, 10μg/ml Galactose-BP/TBSC, 10μg/ml Fucose-BP/TBSC, 10μg/ml GlcNAc-BP/TBSC or 10μg/ml GalNAc- BP/TBSC solution with 5mM $CaCl_2$ or 10mM EDTA was added to each well. The plates were then incubated at 37°C for one hour and were successively washed with TBS/TC as described above. 100μl of the other reagent solution (Vectastain Elite ABC Kit; VECOR) containing avidin and biotinylated horseradish peroxidase (HRP) was added to each well and the plates were incubated at 37°C for 30 minutes. The plates were washed with TBS/TC aforenoted. 100μl of TMB Substrate Solution (TMB Microwell Peroxidase Substrate; Kirkegaard and Perry) was added to each well and the plates were incubated at room temperature for 30 minutes to generate color development. Such color development was then terminated by adding to each well 100μl of 1M phosphoric acid and absorbance of 450nm wavelength light on the subjected polypeptide was determined.

8. As illustrated in the following figure, the tested polypeptide bound all probes and such binding was effectively inhibited by EDTA. These facts clearly indicate that the tested polypeptide is a collectin, *i.e.*, a lectin and binds carbohydrate in Ca^{2+}-dependent manner.

9. FIGURE



OATH/VERIFICATION

10. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief and believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: Signature: N. Wakamiya

Nobutaka Wakamiya

U.S. Patent Application No. 09/763,712
(U.S. Phase of PCT/JP99/04552)

CURRICULUM VITAE OF NOBUTAKA WAKAMIYA, M.D.

[Academic and Professional Background]

March 1980 Graduated from Hirosaki University, Faculty of Medicine, School of Medicine.

July 1980 Joined to Osaka Prefectural Hospital as Resident Pediatrician.

April 1982 Admitted to Doctor`s Course in Department of Pathology (Virus III) at Graduate School of Medicine, Osaka University.

March 1986 Graduated from the Doctor`s Course above. Earned a degree of Doctor of Medicine.

June 1986 Enrolled in Dana-Farber Cancer Institute, Harvard University as a Research Fellow.

Jan. 1988 Joined to Research Institute for Microbial Diseases, Osaka University as an Assistant Professor.

Nov. 2000 Joined Asahikawa Medical College as a Professor and chairman (the present position).

[Specialized Field]

Biochemistry, Immunology and Microbiology

[Membership of Societies Belonged]

The Japanese Biochemical Society
The Japanese Society for Virology
The Japanese Society for Immunology
American Society for Microbiology (ASM)
American Association for Cancer Research (AACR)

[The Present Research Theme]

Biological Functions and Roles of Animal Lectin (Collectin) possessing Collagen Motifs.

[Recent Scientific Japanese Publications on Collectin]

(1) Wakamiya N. and Suzuki Y., `Collectin Families to be acted as Bio-Defensive Lectin`, PROTEIN, NUCLEIC ACID AND ENZYME, Vol.45, No. 5, pp.655-663 (2000).
(2) Wakamiya N. and Suzuki Y., `Novel Hemangioendotheliocyte Scavenger Receptor CL-P1`, SEIKAGAKU, Vol.73, No. 3, pp.205-208 (2001).

[Recent Scientific English Publications on Collectin]

1. Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Wakamiya, N.: Molecular cloning of a novel collectin from liver (CL-L1). J. Biol. Chem. 274(19): 13681-13689, 1999.
2. Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Itabe, H., Suzutani, T., Ogasawara, M., Yoshida, I., Wakamiya, N.: The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. J. Biol. Chem. 276(47): 44222-44228, 2001.